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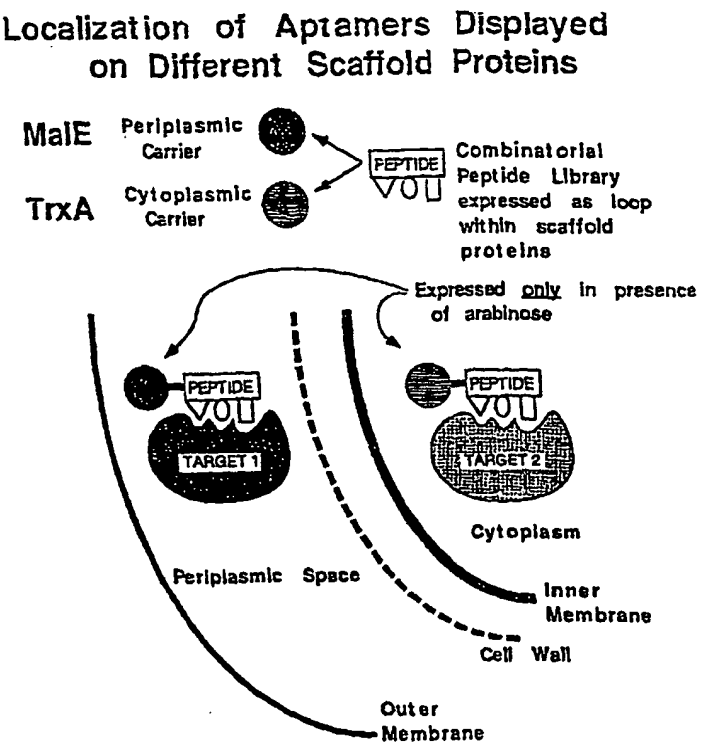
(54) Title: APTAMER BASED BACTERIAL INHIBITION SYSTEMS (ABBIS)

(57) Abstract

The invention features methods of identifying peptide aptamers that bind to essential target molecules and inhibit the viability, growth, or virulence of an organism, methods of isolating compounds that mimic the activity of such peptide aptamers, and peptide aptamers identified by the methods of the invention.



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APTAMER BASED BACTERIAL INHIBITION SYSTEMS (ABBIS)

Statement as to Federally-Sponsored Research

This research has been sponsored in part by NIH R01 Grant AI26289. The government has certain rights to the invention.

Field of the Invention

The field of the invention is antimicrobial drug discovery.

Background of the Invention

The expansion of antimicrobial drug resistance among pathogenic bacteria is a profound threat to human health. Because bacteria and other microbes are becoming resistant to virtually all known antibiotics, any method that facilitates the identification of new antimicrobial molecules is of great social value.

Traditionally, natural and synthetic antimicrobial drugs have been discovered using classic growth inhibitory screens, in which bacteria are exposed to externally added drugs and potential growth inhibition is monitored. However, there are serious limitations to such screens. For example, most bacteria have innate resistance mechanisms, such as effux systems for pumping small molecules out of cells. Moreover, the permeability barrier imposed by the bacterial cell envelope (i.e., bacterial cell wall and membrane layers) prevents drugs from entering many types of bacterial cells, and hence, provides resistance against otherwise toxic drugs.

There are only a few known drug classes that penetrate and remain

within bacterial cells long enough to inhibit an essential bacterial target molecule. Thus, there is a need for fundamentally new approaches of screening for novel antimicrobial compounds that both circumvent innate resistance mechanisms and inhibit growth, viability, or virulence by affecting the activity of novel therapeutic targets.

Summary of the Invention

We have developed a technique, "ABBIS" (Aptamer-Based Bacterial Inhibition System), which provides a means to identify antimicrobial peptide sequences within chimeric proteins (aptamers) that bind to and interfere with the function of target molecules inside microbial cells. These microbial targets are molecules essential for cell growth, viability, virulence, or a combination of these processes. ABBIS is useful for defining potentially novel therapeutic targets and for antimicrobial drug screening assays.

In a first aspect, the invention features a method for identifying an aptamer that inhibits the viability, growth, or virulence of an organism. The method comprises: a) transforming the cells of the organism with an aptamer expression library, wherein expression of aptamers in the library is tightly regulated; b) inducing aptamer expression; c) identifying cells that express aptamers that inhibit the viability, growth, or virulence of the cells, relative to control aptamers that do not inhibit viability, growth, or virulence; and d) isolating aptamer-encoding DNA from cells that express aptamers that inhibit the viability, growth, or virulence of the cells.

In a second aspect, the invention features a method for identifying an aptamer that inhibits the biological function of an essential target molecule. The method comprises: a) transforming cells expressing the essential target molecule with an aptamer expression library, wherein expression of aptamers

in the library is tightly regulated; b) inducing aptamer expression; c) identifying cells that express aptamers that inhibit the viability, growth, or virulence of the cells, relative to control aptamers that do not inhibit viability, growth, or virulence; and d) isolating aptamer-encoding DNA from cells that express aptamers that inhibit the viability, growth, or virulence of the cells.

In a preferred embodiment of the first and second aspects of the invention, the method further includes sequencing the isolated aptamer-encoding DNA.

In a third aspect, the invention features a method for identifying an essential target molecule in an organism, comprising: a) identifying an aptamer that inhibits the biological function of an essential target molecule, wherein identification of the aptamer comprises the method of the second aspect of the invention; b) exposing test samples containing potential essential target molecules to the aptamer ; c) assaying for an interaction between an aptamer and an essential target molecule; and d) determining the identity of an essential target molecule that interacts with the aptamer. In various embodiments, the essential target molecule may be a protein (e.g., the enzyme ThyA), a carbohydrate, a lipid, or a nucleic acid. In other embodiments, the essential target molecule may be identified via its interaction with the inhibitory aptamer through basic biochemical and genetic analyses that are known to those of skill in the art.

In a fourth aspect, the invention features a method for producing a drug screening strain. The method comprises: a) transforming cells expressing an essential target molecule with an aptamer expression library, wherein expression of aptamers in the library is tightly regulated; b) inducing aptamer expression; c) identifying cells that express aptamers at levels that partially inhibit the viability, growth, or virulence of the cells; and d) isolating the

identified cells for use in drug screens.

In one embodiment of the fourth aspect of the invention, the drug screening strain is produced by making the cells hypersensitive to drugs using an aptamer that alters the permeability of the cell. In another embodiment of the fourth aspect of the invention, step (c) is relative to a cell expressing a control aptamer that does not inhibit viability, growth, or virulence.

In a fifth aspect, the invention features a method for producing a drug screening strain. The method comprises: a) producing ThyA/target molecule chimeras by inserting a portion of the target molecule into a permissive location within ThyA; and b) producing cells that express the ThyA/target molecule chimeras.

In the sixth aspect, the invention features a method for detecting a compound that inhibits the biological function of a target molecule. The method comprises: a) inducing a cell from step (d) of the fourth aspect of the invention to express the aptamer at subinhibitory levels; b) exposing the cell to a test compound; and c) assaying for decreased cell viability, growth, or virulence, relative to a cell not exposed to the compound.

In a seventh aspect, the invention features a method for detecting a compound that inhibits the viability, growth, or virulence of a cell. The method comprises: a) inducing a cell from step (d) of the fourth aspect of the invention to express an aptamer at subinhibitory levels; b) exposing the cell to a test compound; and c) assaying for decreased cell viability, growth, or virulence, relative to a cell not exposed to the compound.

In an eighth aspect, the invention features a method for detecting a compound that inhibits the biological function of a target molecule. The said method comprises: a) exposing a cell from step (b) of the fifth aspect of the invention to a test compound; and b) assaying for decreased cell growth on

selective minimal medium lacking thymine.

In a ninth aspect, the invention features a method for detecting a compound that inhibits the biological function of a target molecule. The method comprises: a) exposing a cell from step (b) of the fifth aspect of the invention to a test compound; and b) assaying for increased growth on selective medium containing trimethoprim.

In a tenth aspect, the invention features a method for constructing an aptamer expression library. The method includes: (a) providing a collection of double-stranded random oligonucleotides; (b) providing a plasmid containing a gene encoding a scaffold protein; (c) generating a collection of aptamer-encoding genes by cloning the random oligonucleotides into a permissive site within the gene encoding the scaffold protein, wherein the oligonucleotides are cloned in frame with the coding sequence of the scaffold protein gene, such that each chimeric gene encodes an aptamer, wherein the aptamer consists essentially of a random peptide constrained within a scaffold protein; and (d) ensuring that each aptamer-encoding gene is operably linked to a promoter within an expression vector, wherein the promoter allows tightly regulated expression of the aptamer-encoding gene. In various embodiments, the scaffold protein may be a cytoplasmic protein, a periplasmic protein, or a membrane protein.

In an eleventh aspect, the invention features a method of identifying a compound that binds to an essential target molecule. The method includes: (a) exposing the essential target molecule to an inhibitory aptamer, wherein the aptamer was previously identified using an aptamer expression library in which aptamer expression is tightly regulated; (b) exposing the essential target molecule to a test compound; and (c) measuring the amount of aptamer that is bound to the essential target molecule after the essential target molecule is

exposed to the test compound, wherein a test compound that decreases the amount of aptamer bound to said essential target molecule, relative to the amount of aptamer bound to the identical essential target molecule not exposed to the test compound, and wherein said test compound binds the essential target molecule more strongly than the test compound binds the aptamer, indicates a compound that binds to an essential target molecule.

In various embodiments of the eleventh aspect of the invention, the essential target molecule may be exposed to the aptamer prior to exposing the essential target molecule to the test compound; the essential target molecule may be exposed to the aptamer after exposing the essential target molecule to the test compound; or the essential target molecule may be exposed simultaneously to the aptamer and the test compound.

In another embodiment of the eleventh aspect of the invention, the essential target molecule and the aptamer are located intracellularly. In still another embodiment of the eleventh aspect of the invention, the method may be used to develop small synthetic molecules that are related in structure to a peptide that confers inhibitory activity upon a given aptamer.

In a twelfth aspect, the invention features an aptamer comprising the amino acid sequence set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 10.

In a thirteenth aspect, the invention features a nucleic acid sequence encoding an aptamer comprising the amino acid sequence set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 10.

By "aptamers" is meant random combinatorial peptide sequences, some of which may bind to and inhibit the function of target molecules. Aptamers may be expressed intracellularly by cloning their encoding DNA into

an expression vector, such as pBMT or pBMML2, which are described herein. Aptamers may be expressed within the context of a scaffold protein, such as thioredoxin or maltose binding protein. Such scaffold proteins target the aptamer to a particular subcellular location, such as the periplasmic space. Aptamers are usually between 8 and 70 amino acids in length, although an aptamer may be as small as 4 amino acids or larger than 70 amino acids. For example, aptamers may be, for example, 4-8, 8-16, 9-13, 14-25, 16-34, 16-52, 16-70, 26-30, 31-35, 36-40, 41-45, or 46-50 amino acids in length, or greater than 70 amino acids in length.

By "toxic aptamer" or "inhibitory aptamer" is meant an aptamer that physically interacts with and interferes with the biological function of an essential target molecule, as defined below. Inhibition of viability, growth or virulence may be complete, partial, or conditional, as described below.

By "aptamer expression library" is meant a collection of random oligonucleotide sequences that encodes aptamers, cloned into an expression vector that contains a promoter that directs transcription in the cell type in which the library is to be expressed. Preferably the promoter is an inducible promoter, such as the *araBAD* promoter of *E. coli*, or one of the other inducible promoter systems known for prokaryotic or eukaryotic cells, including tetracyline-inducible, hormone-inducible, metal ion-inducible, or heat shock-inducible promoter systems. Preferably, the aptamers are expressed within the context of chimeric aptamer/scaffold proteins.

By "tightly regulated expression" or "tight transcriptional control" is meant that an aptamer is expressed only when it is desirable to do so. Tightly regulated expression may be achieved through the use of an inducible promoter, e.g., the *E. coli araBAD* promoter described herein.

By "promoter" is meant a minimal sequence sufficient to direct

transcription. Also included are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell type-specific, tissue-specific, temporal-specific, or inducible by external signals or agents.

By "operably linked" is meant that a gene and one or more regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequences.

By "drug screening strain" or "hypersensitized strain" is meant a cell that expresses a toxic or inhibitory aptamer that decreases (but does not completely inhibit) the viability, growth or virulence of the cell, relative to an identical cell that does not express the aptamer. Hence, expression of the aptamer renders the screening strain hypersensitive to compounds that inhibit cell viability, growth or virulence, since these processes are already partially compromised within the screening strain. Use of a screening strain increases the sensitivity of a drug screening assay. The cells of a screening strain may be derived from a variety of organisms, such as bacteria, fungi, parasites, insects, animals, and plants.

By "essential target molecule" or "target molecule" is meant a molecule whose function is required for survival, growth, mitosis/meiosis, or virulence of a cell (e.g, a bacterial, fungal, parasitic, insect, plant, or mammalian cell). A target molecule may also be a molecule whose function is required for the replication, infectivity, or virulence of a virus. A target molecule may be a protein, nucleic acid, lipid, or carbohydrate molecule that interacts with an aptamer in such a way that the biological function of the target molecule is inhibited. Intracellular aptamer/target molecule interactions may be inferred through the detection of a decrease in cell viability, growth or

virulence.

Biological functions of target molecules may include (but are not limited to) participation in enzymatic reactions such as DNA replication, RNA transcription, protein translation, transport of ions, molecules, or other nutrients, or may include functions such as maintainence of cell structures. Interaction of an aptamer with an essential target molecule may be lethal, i.e., prevent a cell (or virus) from surviving (or replicating), growing, or undergoing mitosis/meiosis. Alternatively, interaction of an aptamer with an essential target molecule may allow survival of a cell (or virus), but result in severely diminished growth or virulence; e.g., interaction of an aptamer with an essential target molecule may prevent a cell or virus from expressing components required for virulence, such as toxins, pili, flagella, or other structures. An essential target molecule also may be conditionally essential, i.e., required for viability, growth or virulence under certain conditions, but not under other conditions.

By "determining the identity of an essential target molecule" is meant a process by which the identity of an essential target molecule that interacts with a given aptamer may be ascertained. For example, the target molecule may be purified from cell extracts or expression libraries using an aptamer affinity column, after which the identity of the target molecule may be determined by biochemical analysis (e.g., peptide sequencing), methods of which are known to those skilled in the arts of biochemistry and molecular biology.

By "subinhibitory levels of an aptamer" is meant that an inhibitory aptamer only partially inhibits the biological function of the target molecule with which it interacts. Hence, subinhibitory levels of an aptamer within a cell (such as a screening strain) allows cell survival, although the viability, growth

rate, or virulence may be decreased relative to the same type of cell not expressing the inhibitory aptamer. Moreover, cells expressing subinhibitory levels of aptamers may be hypersensitized to the toxic or inhibitory effects of a second compound to which the cell may be exposed, and therefore, are useful for drug screens. The subinhibitory level of a given aptamer is intrinsic to the aptamer and the cell in which the aptamer is expressed. For example, an aptamer present at a relatively high concentration may only be subinhibitory (as opposed to strongly inhibitory) because its physical association with its target molecule is relatively weak. Alternatively, subinhibitory levels of an strongly inhibitory aptamer may be achieved by allowing only a low level of aptamer expression.

By "expose" is meant to allow contact between a, cell, lysate or extract derived from a cell, or molecule derived from a cell, and an aptamer or test compound. Exposing may be done intracellularly: for example, exposing potential target molecules to an aptamer to by expressing the aptamer within the cell.

By "binds more strongly" is meant that a test compound binds more tightly to an essential target molecule than to an aptamer by at least 10%, preferably by at least 15%, more preferably by at least 20%, still more preferably by at least 30%, yet more preferably by at least 40%, and most preferably by at least 50%. Relative strength of binding may be determined by well known methods, e.g., affinity chromatography, immunoassay, or Scatchard analysis.

By "decrease" is meant a lessening of at least 2-fold (preferably, at least 3-fold, more preferably, at least 5-fold, still more preferably, at least 7-fold, and most preferably, at least 10-fold) in the level of viability, growth, or virulence of a cell or microorganism, in the biological activity (e.g., enzymatic

activity) of an essential target molecule, or in the interaction of an essential target molecule with an aptamer.

By "test compound" is meant a chemical, be it naturally occurring or artificially derived, that is surveyed for its ability to partially or completely inhibit the activity of a target molecule such that the viability, growth, or virulence of a cell is partially or completely inhibited. Test compounds may include, for example, peptides, polypeptides, synthesized organic molecules, naturally occurring organic molecules, nucleic acid molecules, carbohydrates, and components thereof.

By "assaying" is meant analyzing the effect of a treatment administered to cells. The treatment may be, for example, induction of aptamer expression or exposure to a test compound. The analysis may be, for example, for the purpose of detecting altered viability, growth, or virulence resulting from the treatment.

By "permissive location" is meant a region of a protein molecule into which an additional peptide sequence (such as an aptamer sequence or a target molecule sequence) can be inserted, while allowing the protein molecule to retain sufficient biological function to allow cell survival. Permissive location also refers to the corresponding region of a nucleic acid that encodes the above protein molecule, into which an oligonucleotide encoding the above peptide sequence may be inserted.

By "transformation" is meant any method for introducing foreign molecules, such as DNA, into a cell (e.g., a bacterial, yeast, fungal, algal, plant, or animal cell). Lipofection, DEAE-dextran-mediated transfection, microinjection, protoplast fusion, calcium phosphate precipitation, retroviral delivery, electroporation, natural transformation, and biolistic transformation are just a few of the methods known to those skilled in the art which may be

used.

Brief Description of the Drawings

Fig. 1 is a schematic diagram of the ABBIS system.

Fig. 2 is a diagram of the ABBIS-1 vector and a diagram of a constrained 16mer combinatorial peptide within the disulfide loop of TrxA.

Fig. 3 is a diagram of plasmid pBMML2.

Fig. 4 is a diagram of the ABBIS-2 vector and a diagram of a constrained 16mer combinatorial peptide within a permissive loop of MalE.

Fig. 5 is a diagram of an arabinose disk diffusion assay for scoring toxic aptamers.

Figure 6 is a diagram showing growth curves for bacteria expressing the inhibitory aptamers KO1 and KO52, compared to control bacteria not expressing these aptamers.

Fig. 7 is a diagram showing the arabinose-induced trimethoprin resistance and arabinose-induced thymine auxotrophy of *E. coli* clones containing ABBIS-1 vectors that encode anti-ThyA aptamers.

Detailed Description of the Invention

We have developed a technique, "ABBIS" (aptamer-based bacterial inhibition systems), which provides a means to identify toxic chimeric proteins that kill or inhibit the growth or virulence of bacterial cells when expressed intracellularly. Our approach facilitates the isolation of antimicrobial chimeric proteins containing random peptide sequences (aptamers) that bind to and interfere with the function of target molecules inside microbial cells. These microbial target molecules are essential for cell growth, viability, or virulence. Hence, ABBIS is useful for defining potentially novel therapeutic targets, and

peptide inhibitors of such targets.

A schematic outline that illustrates the ABBIS approach is shown in Figure 1. Each ABBIS library is a combinatorial aptamer library composed of constrained random peptides. The peptides are presented as loops of a scaffold protein, which serves to stabilize and display each random peptide within a cell.

Microbial cells, such as those of bacteria, contain physically segregated subcellular compartments. Therefore, in order to access the full range of essential gene products that are potential drug target molecules, it is necessary to target the aptamers to these various subcellular compartments. Hence, each aptamer library is generated using a specific targeting vector, which encodes a scaffold protein that intrinsically localizes to a specific subcellular compartment. For example, below we describe the use of two scaffold proteins, one of which localizes to the cytoplasm (TrxA) of Gram-negative cells and the other, to the periplasm (MalE).

Upon exposure of cells harboring expression-inducible aptamer-encoding plasmids to an inducer molecule (such as arabinose for bacteria, as described below), aptamers are expressed and localized to the appropriate subcellular compartment via scaffold protein localization. Toxic or inhibitory aptamers bind to and inhibit the activity of target molecules that perform essential functions in, for example, the periplasm (Target 1) or the cytoplasm (Target 2) as shown in Fig. 1. Aptamer/target interactions are detected as decreases in cell growth, viability, or virulence.

In a typical ABBIS aptamer/target interaction, a toxic aptamer titrates the activity of its specific target molecule. Hence, ABBIS is also useful as a means of producing aptamer-expressing cell strains for drug screening. In such screeening strains, the activity of a given target molecule is only partially inhibited by an aptamer, resulting in the hypersensitization of the strain to other

drugs that inhibit the target molecule of interest. The use of these hypersensitized screening strains increases the sensitivity of high-throughput screening assays for the identification and isolation of small molecule drugs that inhibit a novel or previously-known target molecule.

Target molecules may be protein, nucleic acid, lipid, or carbohydrate molecules that interact with a toxic aptamer in such a way that the natural function of the target is inhibited. Target molecules, once identified via ABBIS, can be used in conventional high-throughput screens for novel drugs that specifically bind and alter target molecule function in cell-free biochemical screens.

In addition, structural information (i.e., obtained from nuclear magnetic resonance or X-ray crystallographical analysis) of target molecules initially identified using ABBIS allows the application of molecular drug design techniques to create or identify small molecule drugs that bind to and inhibit target molecule function.

Generation of Aptamer-Encoding Sequences

Aptamer-encoding synthetic gene sequences are produced as follows. DNA encoding random stretches of approximately five to thirty amino acids (e.g., combinatorial peptides) are generated by random oligonucleotide synthesis. The synthetic oligonucleotides are cloned into a permissive site within a gene encoding a scaffold protein (See Figs. 2 and 4). The cloning is performed in a way to ensure that the random oligonucleotide sequence is in frame with the scaffold protein coding sequence, resulting in expression of a scaffold protein/aptamer chimera. The scaffold protein-encoding gene is present (or subsequently placed) within an expression vector that is appropriate for the cell type in which the aptamer is to be expressed

(e.g., has an appropriate promoter, origin of replication, etc.). These techniques are readily performed by one of ordinary skill in the art of molecular biology and genetic engineering.

ABBIS Vector Design and Library Construction

We have tested two types of ABBIS targeting vectors: one containing thioredoxin (TrxA) as a scaffold protein, and the other containing maltose binding protein (MalE) as a scaffold protein. These *E. coli* proteins intrinsically localize to the cytoplasmic and periplasmic compartments, respectively, of Gram-negative bacterial cells. Our ABBIS targeting vectors have been engineered to allow insertion of short random peptides into either TrxA or MalE. Presumably, these inserted peptide "loops" are exposed at the surface of the folded chimeric scaffold protein, and are capable of interacting with target molecules.

In theory, any protein that tolerates insertion of random peptides may be used as a scaffold protein for construction of ABBIS libraries. Furthermore, analogous aptamer targeting vectors that express scaffold proteins from Gram-positive bacterial cells, fungi (e.g., yeast), parasites, animal cells, plant cells, etc. may be used to target aptamers to subcellular compartments in these organisms in an analogous fashion.

An important goal of the ABBIS vector design is to ensure that aptamers are expressed only when it is desirable to do so, since toxic aptamer expression causes cell death or growth inhibition. For example, during ABBIS library construction, expression of library-encoded aptamer fusion genes would cause the death of bacterial cells containing toxic aptamers, and as a result, valuable clones could be lost from the library. Hence, aptamer expression must be tightly regulated in order to fully exploit the ABBIS technology.

Chimeric aptamer-encoding genes cloned into the ABBIS vectors described below are under the tight transcriptional control of the *araBAD* promoter. Transcriptional activity of this promoter is strongly repressed in *E. coli* in the presence of glucose, and is strongly induced (more than 300-fold above basal levels) in the presence of 0.4% arabinose. Moreover, intermediate levels of *araBAD* promoter induction are obtained with intermediate arabinose concentrations (e.g., 0.001%). Unlike repressor-based transcription regulatory systems, regulation of the *araBAD* promoter is positive, i.e., transcriptional activation is dependent upon the presence of both the AraC regulatory protein and its activator, arabinose. Hence, spurious expression is less likely to occur than with repressor-dependent systems.

An additional useful feature of ABBIS vectors is the presence of a mobilization sequence derived from plasmid RK2. The mobilization sequence allows highly efficient transfer of ABBIS libraries into various types of bacteria via conjugation. However, any known technique for transformation of DNA into cells, whether natural or artificial, may be used.

The origin of replication for the ABBIS-1 and ABBIS-2 vectors in ColE1 based, and therefore, the vector is maintained at a high copy number in many Gram-negative bacterial species. However, high copy vectors are not necessary, and vectors that are present within cells at low copy numbers also may be used for ABBIS.

It is understood that analogous ABBIS vectors that are appropriate for use with other types of cells, such as bacterial, fungal, parasitic, animal, and plant cells, may be designed by selecting the appropriate plasmid replicons, regulatable expression systems, and scaffold proteins for subcellular targeting of aptamers; appropriate replicons, expression systems, scaffold proteins, and other necessary vector components are known to investigators who are skilled

in the art of molecular biology.

ABBIS-1 library and vector design

The ABBIS-1 library was constructed in plasmid pBMT. This library consists of plasmids that encode aptamers comprising 16 random amino acids (random 16mer peptides). The aptamers are expressed within the context of the disulfide loop of *E.coli* thioredoxin (TrxA), and, hence, are targeted to the cytoplasm. The choice of 16 amino acids as the peptide length for construction of our initial ABBIS libraries was arbitrary: in practice, ABBIS vectors can accept inserts encoding virtually any small peptide.

In order to construct pBMT, the mobilization cassette from pBSL237 (Alexeyev and Shokolenko, *Biotechniques* 19: 22-26; 1995) was cloned into the Cla I site of pBAD18 (Guzman et al., *J. Bacteriol.* 177: 4121-4130; 1995) by ligation of fragments enzymatically rendered blunt-ended, and the resulting plasmid was designated pBADmob. The *trxA* gene from *E. coli* strain XL-1 Blue was cloned by PCR amplification between the *Eco*R I and *Xba* I sites of pBADmob. In order to construct the ABBIS-1 library, oligonucleotides encoding random 16mer peptides were cloned into the unique *Rsr* II site (which begins after encoded amino acid 35 of Trx) of the *trxA* gene.

Fig. 2 shows a schematic diagram of a plasmid isolated from the ABBIS-1 library, plasmid pBMT101Q. The amino acid sequence of the N-terminus of thioredoxin is shown, and the insertion of a random 16mer peptide after amino acid 35 of thioredoxin is denoted by a bracket. The random 16mer peptide shown here comprises a portion of an aptamer that inhibits the function of thymidylate synthase.

ABBIS-2 library and vector design

The ABBIS-2 library was constructed in pBMML2 (Fig. 3). This library consists of plasmids that encode random 16mer peptides expressed within the context of a permissive loop of the *E. coli* maltose binding protein (MalE), and, hence, aptamers are targeted to the periplasmic space.

In order to construct pBMML2, the mobilization cassette from pBSL237 (Alexeyev and Shokolenko, *Biotechniques* 19: 22-26; 1995) was first cloned into the Cla I site of pBAD18 (Guzman et al., *J. Bacteriol.* 177: 4121-4130; 1995) by ligation of fragments enzymatically rendered blunt-ended. The resulting plasmid was designated pBADmob. The *malE* allele containing a short deletion and insertion of a *Bam*H I linker (*malE133*) from plasmid pPD178 (Martineau, et al., *Gene* 113: 35-46; 1992) was amplified by PCR, and the fragment was cloned into the *Sma* I site of pUC19. The resulting plasmid was digested with *Bss*H II and *Bst*X I, and a synthetic oligonucleotide was inserted between these sites to reconstruct the original *malE* sequence, except that the *Bam*H I site was replaced by adjacent *Xho* I and *Xba* I sites. The altered *malE* gene was then excised and cloned between the *Eco*RI and *Hin*d III sites of pBADmob (and is located between base pairs 1628 and 2850 of pBMML2). A large stuffer fragment was cloned between the *Xba* I and *Xho* I sites to create plasmid pBMMLS. The *Xba* I site inserted earlier into *malE* was replaced by a *Bgl* II site by replacing the DNA between the existing *Bgl* II and *Xho* I sites with a synthetic linker. The *Bgl* II site within the original coding sequence of *malE* was eliminated by replacing the 415 base pair fragment between the *Eco*R I and *Bst*X I sites (base pairs 1628 and 2103 of pBMML2) with a PCR product in which the *Bgl* II site was eliminated (without altering the encoded amino acid sequence). The resulting plasmid was designated pBMML2. A large stuffer fragment was then cloned between the remaining *Bgl* II site and the adjacent *Xho* I site to create pBMML2S, which was used for

the construction of the ABBIS-2 library.

As a result of the foregoing cloning steps, plasmid pBMML2 contains a *malE* gene whose sequence differs from the sequence of *malE133* by a silent mutation of the *Bgl* II site, and by the replacement of the region around the *Bam*H I site with the sequence shown below (the *Bgl* II and *Xho* I sites are underlined). In pBMML2S, the "ala" codon shown below is replaced by a large stuffer, which is removed when the library is constructed. The amino acid numbering corresponds to the numbering of the amino acids in the wild-type protein; amino acids without numbers are different from the wild-type sequence.

```
DNA 5'   ATC CCG AGA TCT GCA CTC GAG GGT AAG  3'   (SEQ ID NO: 7)
    3'   TAG GGC TCT AGA CGT GAG CTC CCA TTC  5'   (SEQ ID NO: 8)

protein  ile pro arg ser ala leu glu gly lys       (SEQ ID NO: 9)
aa #     132 133                     143 144
```

In order to construct the ABBIS-2 library, the stuffer region of pBMML2S was released by *Bgl* II/*Xho* I digestion, and oligonucleotides encoding random 16mer peptides were cloned between the *Bgl* II and *Xho* I sites. The random 16mer peptides were inserted into a region of the MalE protein (after amino acid 135) that tolerates large insertions without detrimental effect.

Fig. 4 shows a schematic diagram of a plasmid isolated from the ABBIS-2 library, plasmid pBMML2.12. The bracketed amino acids comprise a portion of an aptamer that has no effect on the growth of the *E. coli* host, and, hence, serves as an ABBIS negative control.

Sequence Complexity of ABBIS Libraries

The ABBIS-1 library consists of 2.7×10^7 plasmids. DNA sequencing of the peptide-encoding region of ten randomly selected ABBIS-1 plasmids showed that all ten plasmids encoded peptides of random sequence. Eight of the plasmids each contained an in-frame insertion consisting of a single oligonucleotide. The ninth plasmid contained an in-frame insertion consisting of two oligonucleotides encoding a 34 amino acid insertion ((2 X 16) + 2, because the sticky ends from the second oligonucleotide generate an additional two codons) that functions as an aptamer. The tenth plasmid contained a single insertion that had a -1 frameshift mutation resulting in premature truncation of the scaffold protein/aptamer chimera. Such truncated aptamers may also have activity (many of them appear to be toxic), but may be more difficult to work with on account of their small size. Similarly, DNA sequencing of two randomly selected plasmids from the ABBIS-2 library showed that these plasmids also encoded peptides of random sequence. Because 16 randomized amino acids can produce approximately 6×10^{20} different peptides, the ABBIS-1 and ABBIS-2 libraries represent only a small fraction of the total universe of random 16mer peptides. It is anticipated that more extensive libraries composed of 10^9 different aptamers are easily obtainable.

Moreover, ABBIS vectors can accept inserts encoding any small peptide. We envision that peptides within the size range of four to fifty amino acids in length will be the most useful, although peptides of other sizes also may be used.

Example I: Isolation of toxic aptamers from the ABBIS-1 library

The ABBIS-1 library plasmid encodes thioredoxin fusion aptamers wherein each of the random peptides is inserted into the thioredoxin gene as an internal fusion after amino acid 35 of thioredoxin. The position of each of these sequences within the protein is the same as the position of the random peptide in pBMT101Q (Fig. 2). Toxic aptamers may be differentiated from non-toxic aptamers by comparing the growth of aptamer-encoding bacterial colonies on medium that induces aptamer expression (in this case, medium containing arabinose) versus control (non-inducing) medium.

Initially, we screened 6100 colonies from the ABBIS-1 library for arabinose-dependent growth inhibition. Colonies were grown on agar medium without arabinose and then transferred by replica plating to agar medium with 0.4% arabinose, on which growth inhibition (induced by aptamer toxicity) was visually scored after 18 hours of incubation.

We obtained 17 clones whose growth was dramatically inhibited by arabinose-mediated aptamer expression (approximately 0.3% of the colonies plated), relative to controls. Based on the size of the ABBIS-1 library we estimate that the library encodes approximately 81,000 toxic aptamer sequences. Expression of the ABBIS-2 library was induced with 0.01% arabinose, and similar results were obtained.

In order to confirm that arabinose-dependent growth inhibition is a function of aptamer expression, we isolated plasmid DNA from bacteria that contained toxic aptamers, transformed the plasmids into naive bacteria, and tested the transformants for arabinose-dependent growth inhibition using disc diffusion experiments (Fig. 5). Bacterial cells expressing either nontoxic control aptamers or toxic aptamers were spread on agar plates. After plating, arabinose-impregnated paper discs were placed at the center of each plate.

After overnight incubation, a lawn of bacteria was observed on each plate. However, the plate that contained bacteria expressing toxic aptamers displayed a clear zone (i.e., less bacterial growth) in the region of the agar into which arabinose had diffused, whereas no clear zone was observed for bacteria not expressing toxic aptamers. This experiment confirms that arabinose-dependent growth inhibition occurs only when toxic aptamers are expressed. Moreover, the disc diffusion method allows the relative toxicity of an aptamer to be determined, since bacteria carrying aptamers of higher toxicity display larger zones of arabinose-induced growth inhibition.

The disc diffusion method also allows the selection of cells resistant to the effects of arabinose. We have observed colonies growing in the zone of inhibition near the arabinose disc. Analysis of these colonies revealed that the bacteria were relatively resistant to arabinose-dependent growth inhibition. Such cells may useful for the characterization of the mechanism of aptamer action. For example, if a given toxic aptamer kills cells by binding to a specific essential molecule (e.g., protein X), mutants that are resistant to the aptamer might contain mutations in protein X that block aptamer binding. These mutations can be mapped, cloned and identified by standard genetic approaches.

We next screened about 4000 colonies for plasmids encoding toxic aptamers by replica plating the colonies from medium lacking arabinose to medium containing 0.4% arabinose. Two candidates were identified, each of which had a single insert, one of which was in frame, and one of which contained a -1 frameshift mutation.

In addition, we also screened 8300 colonies by submaximal induction on 0.005% arabinose and selected 54 candidate toxic aptamer clones for further analysis. Out of 35 clones sequenced, only one contained an in-

frame, single oligonucleotide insert, and this aptamer was not inhibitory. Two of the clones contained an in-frame, double oligonucleotide insert, 31 of the clones contained a -1 frameshift mutation within the insert, and one of the clones contained a -2 frameshift mutation within the insert. There were fifteen clones with a single oligonucleotide insert, eleven clones with a double oligonucleotide insert, three clones with a triple oligonucleotide insert, and three clones with a quadruple oligonucleotide insert. The results of this experiment show that peptides of various sizes may function as toxic aptamers.

Five toxic aptamers that were isolated by replica plating or by submaximal arabinose induction, and inhibited growth of *E. coli* strain SM10, had in-frame oligonucleotide insertions encoding the predicted peptide sequences shown below:

JM7 FRSVCWALTLLGTTFL (SEQ ID NO: 2)

JB63 IRMCLRWSCFCLLTLV (SEQ ID NO: 3)

KO1 LWFTEVRGHGWRYKVG (SEQ ID NO: 4)

KO19 AAYLPRVSMFSWSGCLGPCLLWLFLSLVLCAYLF (SEQ ID NO: 5)

K052 VTLKASVRWYGGGGDTGPRVSWAWVVMLVWAVTL (SEQ ID NO: 6)

Figure 6 shows growth curves for bacteria expressing the inhibitory aptamers KO1 and KO52 shown above, compared to control bacteria not expressing these aptamers. Overnight cultures of *E. coli* SM10 carrying a plasmid encoding either KO1 or KO52 were diluted 10,000-fold into Luria-Bertani medium containing 100 μg/ml ampicillin. The cultures were shaken at 37° C for 2 hours, after which arabinose was added to a final

concentration of 0.1%. Control cultures did not receive arabinose. Samples from experimental and control cultures were removed at the times indicated in Fig. 6 and titers were determined on Luria-Bertani agar plates containing 100 μg/ml ampicillin.

As seen in Fig. 6, arabinose-induced expression of aptamer KO1 appears to be bacteriocidal, and arabinose-induced expression of aptamer KO52 appears to be bacteriostatic. By contrast, arabinose-induced expression of aptamer trxA119, which does not inhibit bacterial growth on solid medium, similarly did not inhibit growth in liquid culture. This experiment shows that aptamers isolated by the methods of the invention may display a range of antimicrobial activity.

The experimental approach described in the previous two paragraphs, i.e., growth of aptamer-expressing bacteria in liquid, arabinose-containing medium, followed by growth on solid medium, may be used to select cells resistant to the effects of arabinose, and, in fact, we have isolated such phenotypic revertants using this approach. Although we found that some of the revertants isolated from growth on arabinose-containing solid medium alone had lost the aptamer-encoding plasmid or had acquired deletions in the aptamer-encoding plasmid, we found that those revertants isolated from arabinose-containing suspension cultures followed by growth on solid medium lacking arabinose had not lost the plasmid, and most had not acquired large deletions. Most of the plasmids isolated from revertants grown in suspension culture, when transformed into a fresh background, again conferred arabinose sensitivity on SM10 *E. coli*. This demonstrates that most mutations leading to loss of arabinose sensitivity in suspension-grown cells were not within the plasmid DNA (and, thus, presumably, were within the chromosomal DNA of the bacteria from which they were isolated). These bacterial mutants may be

useful for genetic studies directed toward identifying the target molecules inhibited by the toxic aptamers.

Example II: Anti-ThyA aptamer isolation

To demonstrate that toxic aptamers isolated using ABBIS inhibit cell growth by inactivating a specific essential target molecule within bacterial cells, we designed an experiment to select for inhibition of a specific essential enzyme. Thymidylate synthase (ThyA) is an essential enzyme in the pathway for biosynthesis of deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP). Mutants defective in ThyA cannot grow on minimal medium that lacks exogenously added thymine; however, the addition of 0.01% thymine to minimal medium allows the growth of ThyA mutants. Mutants deficient in ThyA are resistant to the antibiotic trimethoprim when grown on medium containing thymine. Thus, growth on minimal medium with trimethoprim and thymine provides a positive selection for the ThyA-deficient phenotype.

To isolate aptamers that inactivate the ThyA protein (anti-ThyA aptamers), we plated *E. coli* SM10 containing the ABBIS-1 library on minimal medium containing 0.01% thymine, 1 μg/ml trimethoprim, and 0.01% arabinose, and incubated it for 48 hours at 37° C. We enriched for plasmid-dependent arabinose-dependent trimethoprim resistance by scraping the trimethoprim-resistant colonies from the plates as a pool, isolating plasmid DNA, and re-transforming the pooled plasmids into *E. coli* SM10. Trimethoprim-resistant colonies were then individually chosen, plasmid DNA was isolated, and the individual plasmids were tested for their ability to effect arabinose-dependent trimethoprim resistance and thymine auxotrophy.

Fig. 7 shows an example of an experiment in which anti-ThyA

aptamers were tested for their ability to confer arabinose-induced trimethoprin resistance and thymine auxotrophy. *E. coli* ThyA$^+$ strain SM10 carrying ABBIS-1 plasmids encoding either weakly or strongly inhibitory anti-ThyA aptamers, and control ThyA$^+$ and ThyA$^-$ *E. coli* SM10 strains carrying plasmids that encode inactive aptamers, were uniformly streaked out onto defined medium containing 50 μg/ml ampicillin and various combinations of 0.01% arabinose, 10 μg/ml thymine and 1 μg/ml trimethoprim, as indicated in Fig. 7. Plates were incubated at 37° C for approximately 35 hours.

Fig. 7 shows that anti-ThyA aptamers confer trimethoprim resistance only in the presence of arabinose. Moreover, when arabinose is present and thymine is absent, the strain expressing the weakly inhibitory aptamer grows poorly, and the strain expressing the strongly inhibitory aptamer does not grow at all (the few colonies seen in the strong aptamer sample were confirmed to contain mutant plasmids that had lost their inhibitory properties). Finally, the aptamer-dependent growth inhibition observed on minimal plates containing arabinose could be completely suppressed by the presence of thymine, confirming that the aptamer-dependent growth inhibition was ThyA-specific (the Thy$^+$ control strain grows well under the "Minimal + Arabinose" and "Minimal + Arabinose + Thy" conditions, but its growth is less apparent in the bottom two panels of Fig. 7, because the Thy$^+$ control strain was less-thickly streaked than the other strains).

Fig. 2 shows the deduced peptide sequence of the strongly inhibitory anti-ThyA aptamer (SYLCVTPASQLTEFWG; SEQ ID NO: 1). The DNA sequence encoding this peptide was cloned into the ABBIS-1 vector. This plasmid (pBMT101Q) was transformed into various strains of *E. coli*, and its ability to confer arabinose-dependent trimethoprim resistance and thymine auxotrophy was confirmed.

The series of experiments described above shows that it is possible to isolate aptamers that inhibit bacterial cell growth by interacting with and inhibiting the biological function of an essential target molecule. Furthermore, ABBIS technology also may be used to inhibit eukaryotic enzymes for which there are microbial homologues. For example, it is possible to construct a screening strain of *E. coli* whose growth depends on the human ThyA product, by replacing the *E. coli* thyA gene with the human thyA homologue. Such a strain could then be used in an ABBIS selection to find aptamers that inhibit the human ThyA protein. The aptamers could be used to generate hypersensitive screening strains for high throughput drug screening (see below) or as a starting point for structure-based, molecular drug design. In addition, the aptamers themselves (or modified versions thereof) may be useful pharmaceutical compounds. Drugs discovered using these approaches should be useful for the treatment of disease involving cell proliferation, such as cancer, autoimmune disease, and psoriasis.

The ThyA protein can be converted to a substrate for HIV protease by insertion of a "loop" of HIV substrate peptide into a permissive location within the ThyA protein (ThyA-HIVS). Expression of the HIV protease in bacterial cells whose sole source of ThyA is chimeric ThyA-HIVS results in the $ThyA^-$ phenotype (because the protease cleaves ThyA-HIVS). Thus, the ABBIS system may be used to discover aptamers that inhibit HIV protease (and by analogy, any other protease) by selecting for the arabinose-dependent, $ThyA^+$ phenotype after introducing the ABBIS plasmid library into the strain expressing both the HIV protease and the ThyA-HIVS (or another protease and artificial protease substrate).

As an extension of this approach, ABBIS may be used to discover aptamer inhibitors of any target protein regardless of its origin (e.g.,

mammalian, viral, parasitic) by engineering bacterial strains in which the function of the target protein can be easily scored or modified by a selectable phenotype. Such inhibitory aptamers can then be used as described above as probes for structure-based, molecular drug design.

Alternatively, DNA that encoding inhibitory aptamers identified by ABBIS may be used in gene therapy protocols to inhibit the function of target proteins that cause or are involved in disease processes. Aptamer expression vectors may be engineered for optimal regulation of aptamer expression and subcellular targeting by methods that are known to those who are skilled in the arts of molecular biology, genetic engineering, and gene therapy.

Example III: Construction of an antibiotic hypersensitive screening strain that expresses toxic aptamers at subinhibitory levels

With the exception of the anti-ThyA aptamers, the target molecules of toxic aptamers identified in our preliminary screens of ABBIS libraries are currently unknown. We hypothesized that some of these toxic aptamers might inhibit cell growth by interfering with membrane functions (such as transport) or cell envelope integrity, by inhibiting essential target proteins involved in these cell processes and properties. A "permeabilizing" aptamer might render cells, such as bacteria and fungi, hypersensitive to antibiotics and other antimicrobial agents. Such aptamers would be useful for constructing screening strains that would facilitate the identification of new antimicrobial drugs.

To test this hypothesis, we first determined the concentration of arabinose that would induce subinhibitory expression of a toxic aptamer, thereby decreasing (but not totally inhibiting) the growth of *E. coli* strains expressing toxic aptamers. We screened strains, grown at the determined

arabinose concentration, for their innate resistance to various known antibiotics by disk diffusion assay. As a controls we used the same aptamer-containing strain grown in the absence of arabinose, and a strain expressing a control (non-inhibitory) aptamer.

As shown in Table I below, *E. coli* that encode inhibitory Aptamer 7 display a higher sensitivity to gentamycin when grown in the presence of subinhibitory concentrations of arabinose than when grown in the absence of arabinose; the increased sensitivity results in a relatively larger zone of growth inhibition surrounding gentamycin-impregnated disks. This result shows that toxic aptamers will be useful for constructing drug-hypersensitive screening strains that will enhance the sensitivity of high-throughput drug screens, compared to those screens that employ unmodified bacterial strains. Such hypersensitive strains will be useful for detecting low concentrations of bioactive drugs that are present within complex mixtures.

Screening strains may be constructed using aptamers that inhibit unknown target molecules (such as Aptamer 7), or known target molecules. For example, a screening strain may be constructed using plasmid pBMT101Q (described above), which encodes a strongly inhibitory anti-ThyA aptamer. The appropriate arabinose concentration that induces sub-inhibitory expression of the anti-ThyA aptamer can be readily determined. The strain, when grown under hypersensitizing conditions (e.g., those that induce sub-inhibitory aptamer expression) can be used in screens for drugs that inhibit ThyA.

Table I: Effects of aptamer expression on growth inhibition by gentamicin

Inhibitory aptamer	Arabinose concentration 0	.005%
7	18 mm	24 mm
31	17 mm	20 mm
63	18 mm	24 mm
control	20 mm	20 mm

Legend

Exponentially growing cultures of *E.coli* (strain SM10) containing plasmids that encode the indicated aptamers were spread onto LB agar with ampicillin, with or without 0.005% arabinose (0.005% arabinose induces sub-maximal levels of aptamer expression). A disc impregnated with 10 μg of gentamicin was placed on the plate, which was then incubated overnight. The zone of inhibition around the gentamicin disc was measured, and the diameter is shown above. The larger the zone, the lower the concentration of gentamicin required to inhibit growth. The control plasmid encodes an aptamer that is not toxic to the cells.

Example IV: Selection for aptamers that inhibit the function of virulence factors, improvement of aptamers and conversion to drugs, and identification of aptamer targets by genetic and biochemical approaches

As described above, toxic ABBIS aptamers may be identified by screening for general bacterial growth inhibition, or by screening for inhibition of a specific essential target molecule such as ThyA. Moreover, useful ABBIS aptamers may be identified using target molecules that are not essential for bacterial growth and survival, or that are essential only under certain conditions. Such targets may, however, play important roles in infection and disease.

For example, bacterial virulence factors constitute a group of proteins (e.g., toxins, enzymes, or regulatory proteins) or structures (e.g., pili, flagella, capsules, or secretion systems) that are not essential for bacterial viability on artificial media. However, virulence factors frequently are essential for growth and survival in host tissues. An antimicrobial compound that interferes with the biological function of a virulence factor would have clinical value in the treatment of infection. Hence, ABBIS may be used for the isolation of aptamers that inhibit nonessential or conditionally essential target molecules, such as bacterial virulence factors, by screening for arabinose-dependent loss of virulence. *In vitro* and *in vivo* screens for virulence factor function are known to those of skill in the art of microbiology.

The desired activity of a given aptamer may be improved by performing repeated cycles of random mutagenesis of an aptamer coding sequence followed by selection of the desired activity under decreasing arabinose concentrations. This mutational modification and selection of aptamers also provides a means by which to define critical residues that are

responsible for aptamer activity.

Once the minimal peptide sequence of an inhibitory aptamer is defined, peptide derivatives that contain the minimal inhibitory sequence plus additional chemical moieties may be synthesized. Such functional groups (e.g., cyclic bonds, peptide bond mimics, etc.) may be employed, for example, to stabilize a synthetic peptide, constrain it conformationally, or stimulate its uptake by bacterial cells. The addition of such moieties can enhance the toxic activity of an aptamer, thus increasing its usefulness as an antimicrobial compound.

Once an aptamer is identified that inhibits the function of an essential molecule or bacterial virulence factor, standard biochemical and genetic analyses may be used to determine the target molecule affected by the aptamer. For example, host bacteria can be chemically mutagenized prior to introduction of a plasmid encoding a known aptamer. Mutants that resist the action of a given aptamer can be selected, and the gene that confers resistance against an aptamer can be cloned and mapped by standard genetic complementation methods. DNA sequencing can be used to identify the mutations that allowed the target molecule to escape from the aptamer's inhibitory activity, assuming that resistance to an aptamer results from mutations in the aptamer target molecule itself.

Biochemical binding of an aptamer to its target molecule may be assessed by passing cell extracts over an aptamer-derivatized column. Alternatively, radiolabelled or enzyme-labelled aptamers could be used as probes to isolate target molecules (for example, using a protocol analagous to a Western blot). Extracts of mutant, aptamer-resistant cells would be useful controls in such experiments. Once the target of the inhibitory aptamer is identified, standard drug discovery methods and high throughput screening can

be used to find small molecule drugs that inhibit the target molecule.

The sequences of a combinatorial peptide that are crucial for the inhibitory activity of a given aptamer can be determined by construction of substitution or deletion mutations, followed by structure-function analyses, and analyses of aptamer/target interactions. Ultimately, crystallization of complexes between an aptamer-derived peptide and its target molecule will provide the most detail for each aptamer/target interaction. Such information would accelerate the synthesis of aptamer analogs (i.e., peptide mimetics) that are useful as antimicrobial drugs.

Example V: Selection for aptamers that inhibit or stimulate the PhoBR signal transduction pathway

The PhoBR signal transduction pathway regulates phosphate concentration-dependent gene expression (for review, see B. L. Wanner, *in: Metal Ions in Gene Regulation*, S. Silver and W. Walden, eds., Chapman and Hall, 1997, pp. 104-128). PhoBR is a two-component pathway requiring a phosphate sensor molecule, PhoR, and a transcriptional activator molecule, PhoB. In response to a low extracellular phosphate concentration, PhoR, a transmembrane histidine kinase, phosphorylates itself at a conserved cytoplasmic histidine residue, after which it transfers the phosphoryl group to an aspartate residue on PhoB. Phosphorylation of PhoB leads to transcriptional activation of at least eleven phosphate-regulated genes.

PhoR also possesses a phosphatase activity that deactivates phospho-PhoB when extracellular phosphate increases. This negative regulation is dependent on PstSCAB, a phosphate-specific transporter, and PhoU, a peripheral membrane protein. (In addition, the system is regulated by two other phosphate-independent signals, acetyl phosphate and CreC, which

are not relevant in the system described below, because our strain is deleted for *creC*, *pta*, and *ackA*, which prevents cross-talk from the other pathways.)

We have chosen to employ the PhoBR pathway as a model for isolating aptamers that inhibit potential therapeutic targets, because this pathway is representative of many other two-component signaling pathways that regulate important cellular processes, such as chemotaxis, expression of virulence factors, and antibiotic resistance.

In order to search for aptamers that inhibit negative regulators of the PhoB-PhoR system, we introduced the ABBIS library into the reporter strain BW25256 (P_{phnC}-*lacZ* Δ*creC* Δ*(ackA pta)*Δ*ara rpsL*). We measured expression of two genes activated by low extracellular phosphate: P_{phnC}-*lacZ*, a chimeric reporter gene composed of a *lacZ* coding region under the transcriptional regulation of the PhoB-dependent *phnC* promoter, and *phoA,* a second PhoB-dependent gene. When extracellular phosphate levels are high (greater than about 4 μm or so in this system), neither P_{phnC}-*lacZ* nor *phoA* is expressed.

As shown in the genotype above, BW25256 is deleted for the arabinose operon, and this strain cannot break down arabinose. As a result, of the *araBAD* promoter that regulates aptamer expression from the ABBIS-1 library vector occurs at very low arabinose concentrations. We plated the cells on M9 minimal medium containing lactose as a carbon source, high phosphate (about 64 μM, which inhibits the PhoBR pathway, thereby suppressing expression of the P_{phnC}-*lacZ* and *phoA* genes), 0.05% arabinose to induce aptamer expression, and XP (5-bromo-4-chloro-3-indolyl phosphate) as an indicator of PhoA (alkaline phosphatase) expression. As expected, most of the cells failed to grow on this medium, since the high phosphate concentration shuts off expression of LacZ (beta-galactosidase), which is required for growth

on lactose as a sole carbon source. However, if an aptamer were to interfere with the activity of the PhoR phosphatase, PstSCAB phosphate transporter, or PhoU, the cells would express both LacZ and PhoA in the presence of arabinose, and would form blue colonies due to hydrolysis of XP.

We enriched for bacterial clones expressing aptamers that allow arabinose-dependent PhoA and LacZ expression by scraping the initial colonies from the plates as a pool, isolating plasmid DNA, re-transforming the pooled plasmids into BW25256, and plating the transformed bacteria onto the same selective medium. Blue colonies were then individually chosen and tested for their ability to effect arabinose-dependent PhoA and LacZ expression. We isolated many blue clones that showed plasmid-linked, arabinose-dependent expression of both LacZ and PhoA on high-phosphate medium. Sequence analysis revealed that all of the plasmids that gave this phenotype encoded an aptamer containing a random peptide of the sequence CWVDKPWIGQYLCLCE (SEQ ID NO: 10). Expression of this aptamer in *E. coli* grown in high phosphate medium led to a 15- to 70-fold induction of expression of alkaline phosphatase. One can determine the target of the PhoBR pathway aptamer by screening for a reversal of the aptamer's effect upon overexpression of genes encoding potential targets of this aptamer.

Identification of compounds that mimic the binding properties of inhibitory aptamers

Once essential target molecule/inhibitory aptamer binding pairs are identified, these reagents may be used in high-throughput assays to isolate compounds that mimic a given apatamer's ability to bind a given essential target molecule. Compounds identified by such approaches may have utility as therapeutic agents in inhibiting the viability, growth, or virulence of undesired

cell or microorganisms.

Enzyme-linked immunosorbant assays

Enzyme-linked immunosorbant assays (ELISAs) are easily incorporated into high-throughput screens designed to test large numbers of compounds for their ability to mimic the binding activity of a given inhibitory aptamer. Changes in the level of interaction between an essential target molecule and its aptamer binding partner may reflect the ability of a test compound to mimic the aptamer's binding activity. Protocols for ELISA may be found, for example, in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1998.

In one embodiment, samples containing an essential target molecule of interest are loaded onto the wells of microtiter plates coated with an aptamer known to bind to and inhibit the biological activity of the essential target molecule. Unbound antigen is washed out, and an antibody that specifically recognizes the essential target molecule, coupled to an agent to allow for detection, is added. Agents allowing detection include alkaline phosphatase (which can be detected following addition of colorimetric substrates such as *p*-nitrophenolphosphate), horseradish peroxidase (which can be detected by chemiluminescent substrates such as ECL, commercially available from Amersham) or fluorescent compounds, such as FITC (which can be detected by fluorescence polarization or time-resolved fluorescence). The amount of antibody binding, and hence the relative amount of aptamer bound to the essential target molecule in a sample, is easily quantitated on a microtiter plate reader. It is understood that appropriate controls for each assay are always included as a baseline reference. A decrease in the amount of aptamer/essential target molecule binding suggests that a test compound may bind to the essential

target molecule.

A positive assay result, that is, identification of a compound that mimics the binding of a inhibitory aptamer to an essential target molecule, is indicated by a decrease of at least 2-fold (preferably, at least 3-fold, more preferably, at least 5-fold, still more preferably, at least 7-fold, and most preferably, at least 10-fold) in the level of essential target molecule/aptamer complexes.

To confirm that a test compound disrupts an essential target molecule/aptamer interaction as of a result of its binding to the essential target molecule, rather than to the aptamer, the test compound may be bound to a solid support, and its binding affinity for the essential target molecule versus the aptamer may be determined. A test compound that disrupts the essential target molecule/aptamer interaction by binding the essential target molecule will have a higher affinity for the essential target molecule than for the aptamer.

High-throughput assays for the purpose of identifying compounds that mimic the binding activity of a given aptamer isolated by the methods of the invention can be performed using aptamers and/or essential target molecules within cell lysates, or as purified or partially-purified molecules. It is understood that variations of the assay described above, or other binding assays that will be apparent to those of ordinary skill in the art, may be employed to identify compounds that mimic the binding activity of an inhibitory aptamer to its cognate essential target molecule. For, example, in one variation of the ELISA assay described above, the microtiter wells are coated with the essential target molecule and the level of essential target molecule/aptamer interaction is detected by using an antibody that specifically binds the aptamer.

Compounds that are identified as having the ability to bind essential target molecules may be tested for their ability to inhibit the biological activity (e.g., enzymatic activity) of such molecules or to inhibit the growth, viability, or virulence of a cell or microorganism by methods that are known to those of ordinary skill in the art.

Test Compounds

In general, novel drugs that bind to and inhibit the biological activity of essential target molecules or inhibit the viability, growth, or virulence of an organism are identified from large libraries of both natural products or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch

Oceangraphics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their ability to bind to and inhibit the biological activity of essential target molecules or inhibit the viability, growth, or virulence of an organism should be employed whenever possible.

When a crude extract is found to inhibit the biological activity of one or more essential target molecules, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract having the ability to bind to and inhibit the biological activity of at least one essential target molecule or having the ability to inhibit the viability, growth, or virulence of an organism. The same assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful agents for treatment are chemically modified according to methods known in the art. Compounds identified as being of therapeutic value may be subsequently analyzed, if appropriate, using one of many animal models known to be useful for

pharmaceutical testing.

Therapy

The compounds (inhibitory aptamers and small molecule compounds that mimic the binding properties of inhibitory aptamers) identified using any of the methods disclosed herein, may be administered to patients or experimental animals with a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer such compositions to patients or experimental animals. Although intravenous administration is preferred, any appropriate route of administration may be employed, for example, parenteral, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for antagonists or agonists of the invention include ethylene-vinyl acetate copolymer particles, osmotic pumps,

implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

Other Embodiments

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth, and follows in the scope of the appended claims.

What is claimed is:

1. A method for identifying an aptamer that inhibits the viability, growth, or virulence of an organism, said method comprising:

a) transforming the cells of said organism with an aptamer expression library, wherein expression of aptamers in said library is tightly regulated;

b) inducing aptamer expression;

c) identifying cells that express aptamers that inhibit the viability, growth, or virulence of said cells, relative to control aptamers that do not inhibit said viability, growth, or virulence; and

d) isolating aptamer-encoding DNA from cells that express aptamers that inhibit the viability, growth, or virulence of said cells.

2. A method for identifying an aptamer that inhibits the biological function of an essential target molecule, said method comprising:

a) transforming cells expressing said essential target molecule with an aptamer expression library, wherein expression of aptamers in said library is tightly regulated;

b) inducing aptamer expression;

c) identifying cells that express aptamers that inhibit the viability, growth, or virulence of said cells, relative to control aptamers that do not inhibit said viability, growth, or virulence; and

d) isolating aptamer-encoding DNA from cells that express aptamers that inhibit the viability, growth, or virulence of said cells.

3. A method for identifying an essential target molecule in an organism, said method comprising:

a) identifying an aptamer that inhibits the biological function of an

essential target molecule, wherein identification of said aptamer comprises:

(i) providing a population of cells expressing said essential target molecule and transformed with an aptamer expression library, wherein expression of aptamers in said library is tightly regulated;

(ii) inducing aptamer expression;

(iii) identifying a cell that expresses an aptamer that inhibits the viability, growth, or virulence of said cell, relative to a control aptamer that does not inhibit said viability, growth, or virulence;

(iv) isolating aptamer-encoding DNA from said cell that expresses said aptamer that inhibit the viability, growth, or virulence of said cell; and

(v) sequencing the isolated aptamer-encoding DNA;

b) exposing test samples containing potential essential target molecules to said aptamer;

c) assaying for an interaction between an aptamer and an essential target molecule; and

d) determining the identity of said essential target molecule.

4. A method for producing a drug screening strain, said method comprising:

a) transforming cells expressing an essential target molecule with an aptamer expression library, wherein expression of aptamers in said library is tightly regulated;

b) inducing aptamer expression;

c) identifying a cell that expresses an aptamer at a level that partially inhibits the viability, growth, or virulence of said cell;

d) isolating said identified cell for use in drug screens.

5. A method for producing a drug screening strain, said method comprising:

a) producing a ThyA/target molecule chimera by inserting a portion of said target molecule into a permissive location within ThyA;

b) producing a cell that expresses said ThyA/target molecule chimera.

6. A method for detecting a compound that inhibits the biological function of a target molecule, said method comprising:

a) inducing a cell from a drug screening strain to express an aptamer at a subinhibitory level, wherein:

(i) said cell expresses an essential target molecule;

(ii) expression of said aptamer in said cell is tightly regulated; and

(iii) expression of said aptamer in said cell is at a level that partially inhibits the viability, growth, or virulence of said cell;

b) exposing said cell to a test compound; and

c) assaying for decreased cell viability, growth, or virulence, relative to an identical cell not exposed to said compound.

7. A method for detecting a compound that inhibits the viability, growth, or virulence of a cell, said method comprising:

a) inducing a cell from a drug screening strain to express an aptamer at a subinhibitory level, wherein:

(i) said cell expresses an essential target molecule;

(ii) expression of said aptamer in said cell is tightly regulated; and

(iii) expression of said aptamer in said cell is at a level that partially inhibits the viability, growth, or virulence of said cell;

b) exposing said cell to a test compound; and

c) assaying for decreased cell viability, growth, or virulence, relative to an identical cell not exposed to said compound.

8. A method for detecting a compound that inhibits the biological function of a target molecule, said method comprising:

a) exposing a cell from a drug screening strain to a test compound, wherein said cell expresses a ThyA/target molecule chimera; and

b) assaying said cell for decreased cell growth on selective minimal medium lacking thymine, wherein decreased cell growth on said selective medium lacking thymine, compared to the growth of an identical cell not exposed to said test compound, indicates a compound that inhibits the biological function of said target molecule.

9. A method for detecting a compound that inhibits the biological function of a target molecule, said method comprising:

a) exposing a cell from a drug screening strain to a test compound, wherein said cell expresses a ThyA/target molecule chimera; and

b) assaying for increased growth on selective medium containing trimethoprim, wherein increased cell growth on said selective medium lacking thymine, compared to the growth of an identical cell not exposed to said test compound, indicates a compound that inhibits the biological function of said

target molecule.

10. An aptamer comprising the amino acid sequence set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 10.

11. A nucleic acid sequence encoding an aptamer comprising the amino acid sequence set forth in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, or SEQ ID NO: 10.

12. A method for constructing an aptamer expression library, said method comprising:

a) providing a collection of double-stranded random oligonucleotides;

b) providing a plasmid comprising a gene encoding a scaffold protein;

c) generating a collection of aptamer-encoding genes by cloning said random oligonucleotides into a permissive site within said gene encoding said scaffold protein, wherein said oligonucleotides are cloned in frame with the coding sequence of said scaffold protein gene, such that each chimeric gene encodes an aptamer, wherein said aptamer consists essentially of a random peptide constrained within a scaffold protein; and

d) ensuring that each aptamer-encoding gene is operably linked to a promoter within an expression vector, wherein said promoter allows tightly regulated expression of said aptamer-encoding gene.

13. The method of claim 12, wherein said scaffold protein is a

cytoplasmic protein, a periplasmic protein, or a membrane protein.

14. A method of identifying a compound that binds to an essential target molecule, said method comprising:

a) exposing said essential target molecule to an inhibitory aptamer, wherein said aptamer was previously identified using an aptamer expression library in which aptamer expression is tightly regulated;

b) exposing said essential target molecule to a test compound; and

c) measuring the amount of aptamer that is bound to said essential target molecule after said essential target molecule is exposed to said test compound,

wherein a test compound that decreases the amount of aptamer bound to said essential target molecule, relative to the amount of aptamer bound to the identical essential target molecule not exposed to said test compound, and wherein said test compound binds said essential target molecule more strongly than said test compound binds said aptamer, indicates a compound that binds to an essential target molecule.

15. The method of claim 14, wherein:

said essential target molecule is exposed to said aptamer prior to exposing said essential target molecule to said test compound;

said essential target molecule is exposed to said aptamer after exposing said essential target molecule to said test compound; or

said essential target molecule is exposed simultaneously to said aptamer and said test compound.

16. The method of claim 14, wherein said essential target molecule

and said aptamer are located intracellularly.

17. The method of claim 1 or 2, further comprising sequencing said isolated aptamer-encoding DNA.

18. The method of claim 4, wherein step (c) is relative to a cell expressing a control aptamer that does not inhibit viability, growth, or virulence.

SEQUENCE LISTING

<110> President and Fellows of Harvard College

<120> APTAMER BASED BACTERIAL INHIBITION SYSTEMS (ABBIS)

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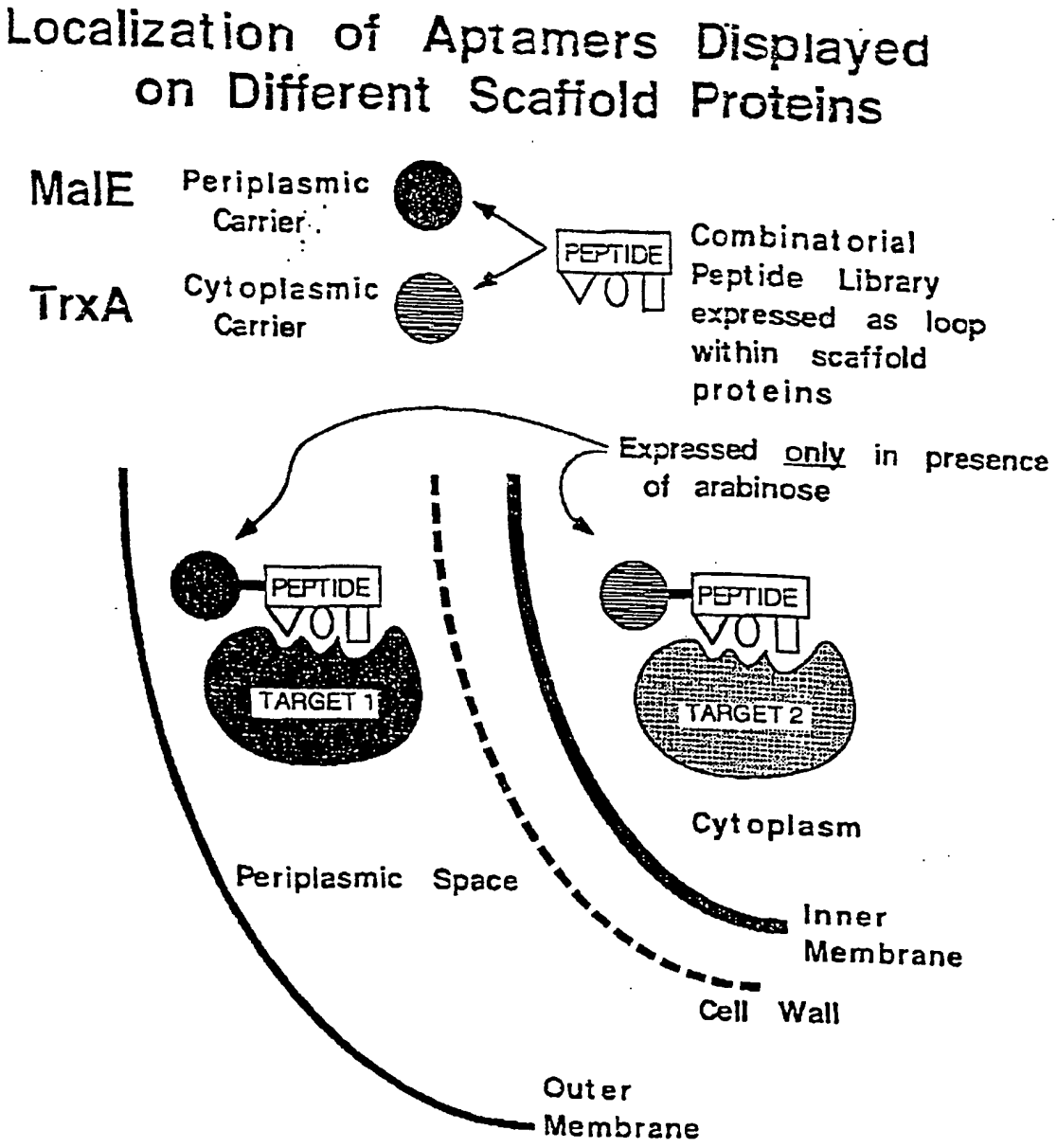
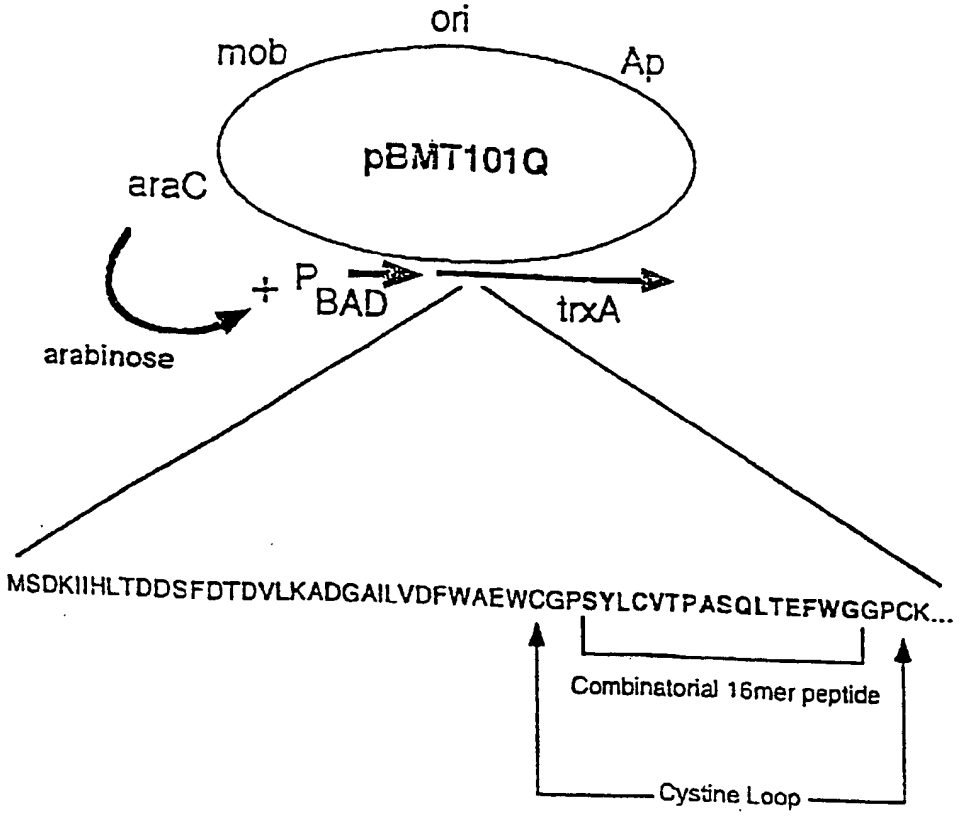

Localization of Aptamers Displayed on Different Scaffold Proteins
MalE
Periplasmic Carrier
TrxA
Cytoplasmic Carrier
PEPTIDE
Combinatorial Peptide Library expressed as loop within scaffold proteins
Expressed only in presence of arabinose
PEPTIDE
TARGET 1
PEPTIDE
TARGET 2
Cytoplasm
Periplasmic Space
Inner Membrane
Cell Wall
Outer Membrane

FIG. 1

Example of Aptamer Encoding Plasmid from ABBIS-1 Library



Constrained Conformation
Via
TrxA Disulfide Loop

FIG. 2

pBMML2 -> Graphic Map

DNA sequence 6106 b.p. atctgataggtg ... tgacagcttatc circular

pBMML with the Bgl II site mutagenized to ablate it, and the Xba I site replaced with a Bgl II site.

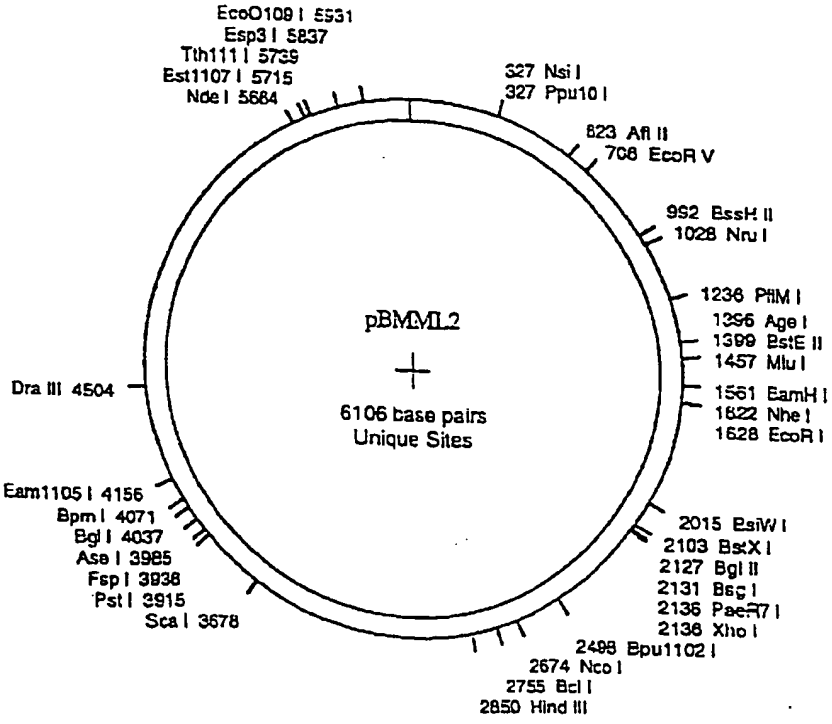
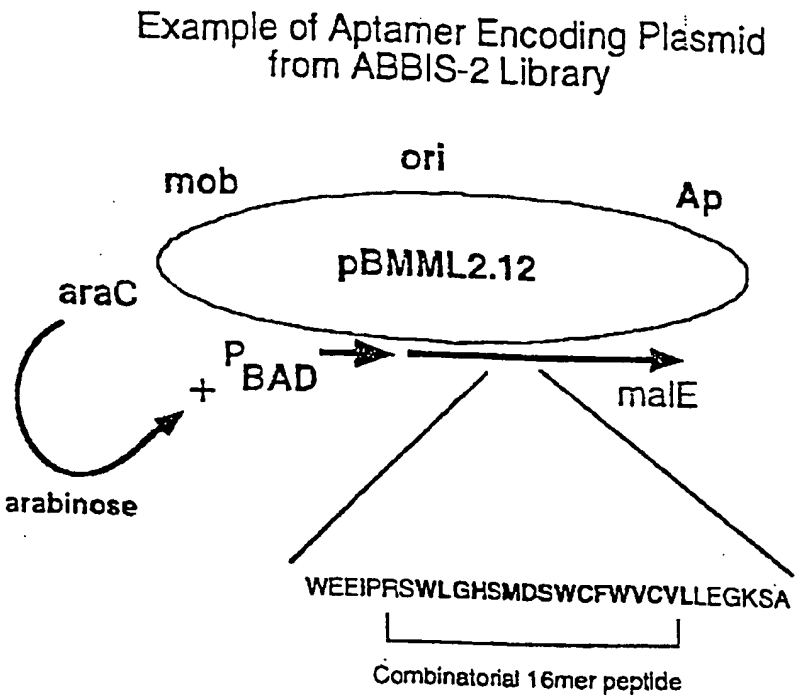
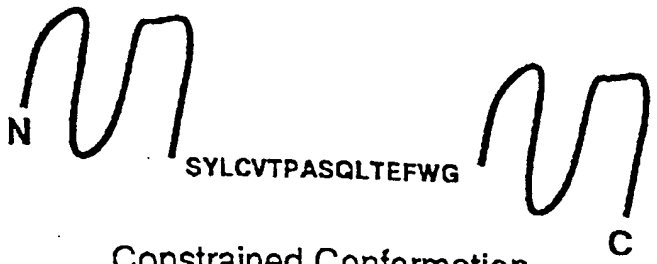


FIG. 3

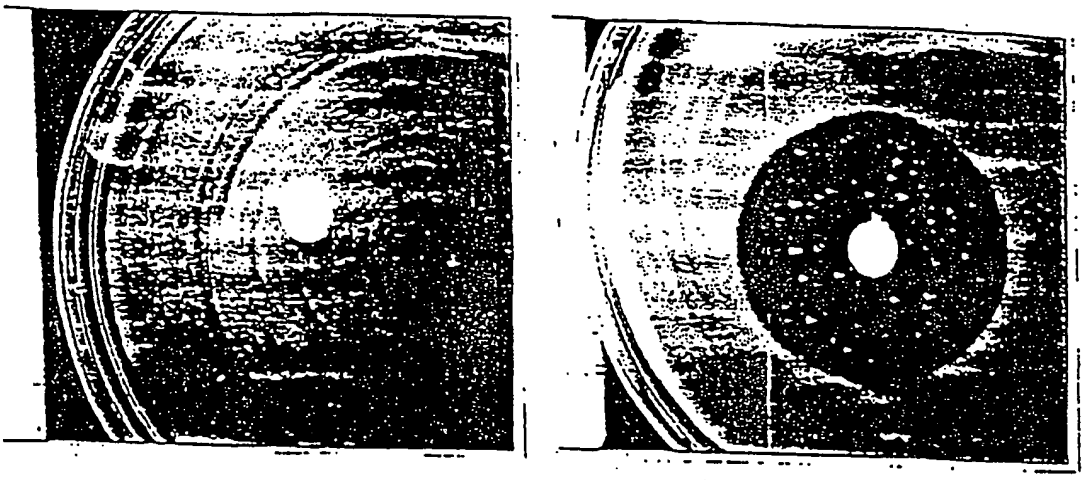




Constrained Conformation
Via
MalE Tertiary Structure

FIG. 4

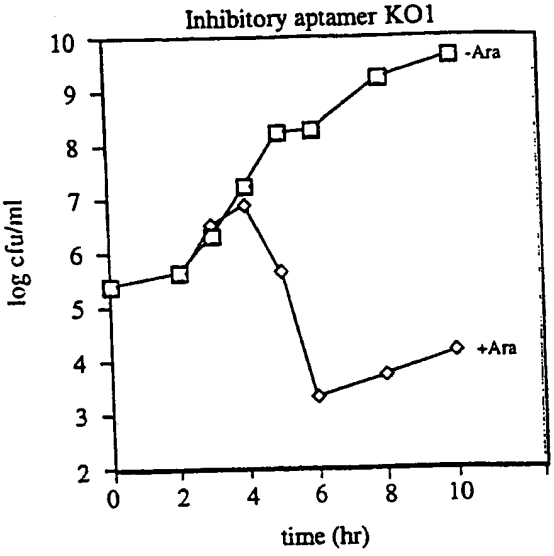
Arabinose Disk Diffusion Assay for Scoring Toxic Aptamers*



Strain Carrying Control Aptamer Strain Carrying Toxic Aptamer

*Both disks were soaked in 10% arabinose

FIG. 5


Inhibitory aptamer KO1
log cfu/ml
time (hr)
-Ara
+Ara
10
9
8
7
6
5
4
3
2
0
2
4
6
8
10

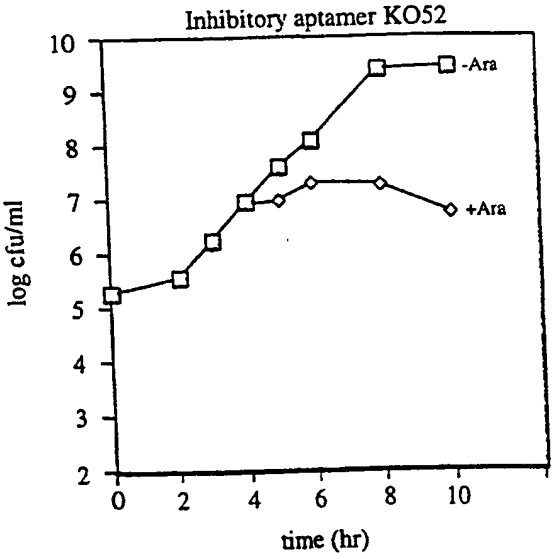
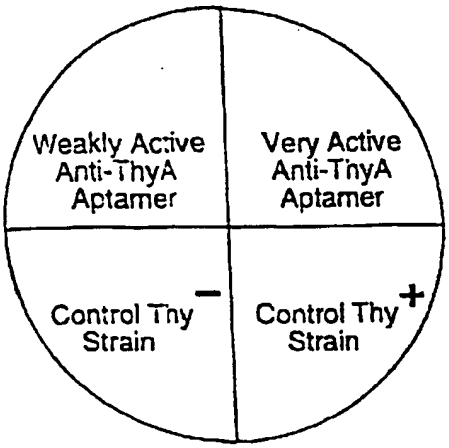
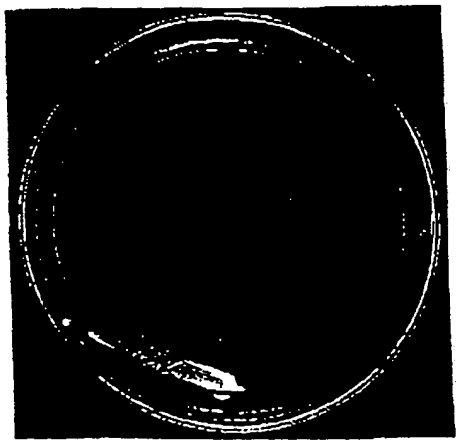

Inhibitory aptamer KO52
log cfu/ml
time (hr)
-Ara
+Ara
10
9
8
7
6
5
4
3
2
0
2
4
6
8
10

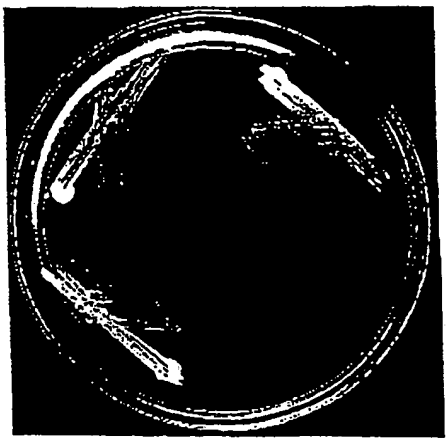
FIG. 6



Minimal + Glucose



Trimethoprim



Trimethoprim + Arabinose

Minimal + Arabinose

Minimal + Arabinose + Thy

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06466

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/70, 1/68; C12N 15/00; C07K 5/00
US CL : 435/6, 5, 4, 471; 530/326
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 5, 4, 471; 530/326

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS, BIOSIS
search terms; aptamer, random combinatorial peptide library, scaffold protein, thioredoxin or maltose binding protein

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P --- A, P	US 5,739,309 A (DATTAGUPTA et al) 14 April 1998, column 13, lines 1-39.	1, 12-13, 17 --- 10
A, P	US 5,780,449 A (BRACHT et al) 14 July 1998, entire document.	1, 10, 12-13, 17
Y	US 5,270,170 A (SCHATZ et al) 14 December 1993, column 2, lines 25-63.	1, 12-13, 17
A	US 5,432,018 A (DOWER et al) 11 July 1995, entire document.	1, 10, 12-13, 17
A	US 5,582,981 A (TOOLE et al) 10 December 1996, entire document.	1, 10, 12-13, 17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
15 JUNE 1999	12 JUL 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer T. WESSENDORF Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06466

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1, 10, 12, 13, 17 drawn to a method for identifying aptamers that inhibits the viability, growth and virulence of an organisms.
Group II, claim(s) 2, 17, drawn to method of identifying aptamer that inhibits the biological function of an essential target molecule.
Group III, claim(s) 3, drawn to a method of identifying an essential target molecule in an organism.
Group IV, claim(s) 4, 18, drawn to a method for producing a drug screening strain.
Group V, claim(s) 5, drawn to a method for producing drug screening strain by producing a specific Thy A/target molecule.
Group VI, claim(s) 6, drawn to a method for detecting a compound that inhibits the biological function of a target molecule.
Group VII, claim(s) 7, drawn to a method for detecting a compound that inhibits the growth, viability and virulence of a cell.
Group VIII, claim(s)8, drawn to a method for detecting a compound that inhibits the biological function of a target molecule.
Group IX, claim(s) 9, drawn to a method for detecting a compound that inhibits the biological function of a target molecule by exposing a cell from a drug screening strain.
Group X, claim(s)11, drawn to nucleic acid.
Group XI, claim(s) 14-16, drawn to method of identifying a compound.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the method of Group II requires inhibition of a biological function of a target molecule which include enzyme reactions like DNA replication.

The inventions listed as Groups I and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the method of Group III relates to a portion of the organism rather than the whole organism as recited in the invention of Group I.

The inventions listed as Groups (I-III, XI) and Group (IV or V) do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the method of Groups (I-III) relate to a method of screening to identify an aptamer that inhibits the biological function of a target molecule as recited in Group II or an organism recited in Group I i.e., drawn to different methods of screening for compounds exhibiting different biological functions or effects. Group IV or V relates to a method of producing i.e., making of a drug screening strain.

The inventions listed as Groups (I-III, XI) and Group (VI- IX) do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the method of Groups (I-III) relate to a method of screening to identify an aptamer that inhibits the biological function of a target molecule as recited in Group II or an organism recited in Group I. Group VI-IX relate to a method of detecting a compound as obtained from an aptamer library.

The inventions listed as Groups (I-XI) and X do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the method of Group IV requires positive, manipulative process steps for screening, detecting or identifying a compound. The nucleic acid of Group X can be used as a probe.

The inventions listed as Group IV and Group V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the method of Groups IV do not require a chimera as recited in Group V.

The inventions listed as Groups VI and VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the method of Group II requires inhibition of a biological function of a target molecule which include enzyme reactions like DNA replication.

The inventions listed as Group VI and Group VIII or IX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the method of Groups VI do not require a chimera as recited in Group VIII or IX.

This application contains claims directed to more than one species of the generic invention. These species are deemed

to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

The species recited in claim 10 as follows:

Peptides having the amino acid sequences: Seq. ID.1 to Seq. ID. 10.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: each of these species lack the same or corresponding structural features as each of these species are produced from the random peptide library. Therefore, each of these species can exhibit functional differences.

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